Isothermal Titration Calorimeter

1. If you have no idea of your system's binding constant, suggestions for starting concentrations of protein and ligand solutions are given in Section 6.5 of the VP-ITC User's Manual. (available online at www.microcalorimetry.com)

2. Protein and ligand solutions need to be in the same buffer so no heat of dilution will be generated when they mix. If possible, dialyze protein and ligand solutions against the buffer they're in and use the dialysis buffer in the reference cell. The Sample Preparation Tips section of the ITC's webpage discusses several parameters. DTT and TCEP are better avoided in your buffer. See Section 6.3 of the User's Manual for reasons and possible substitutes.

3. Turn on the computer first. Log on as ITC User using the password: itc. Then turn on the instrument itself. Switch is a rocking toggle located at the upper left on the back. Open the VPViewer 2000 software at least 30 seconds after turning on the instrument. Otherwise, there won't be a Y-axis scale chosen under the Setup/Maintenance tab or constants loaded under the Constants tab. Finally, turn on power to the Thermo-Vac. Switch is a rocking toggle at the upper right on the back.

4. Solutions need to be degassed first since air coming out of solution will also introduce spurious heat. Set the temperature of the Thermo-Vac unit to 3-4° below your experimental run temp. since it's much quicker for the machine to heat the sample a little than to cool it. (Note: there is a missing decimal point on the temperature display. 24° looks like 240°.)

   Put 2.5 ml of protein solution in a plastic vial with a stir bar and put ~500ul of ligand solution in a small glass tube. Both can be degassed at the same time. Place the vacuum cap over the vacuum chamber and tighten the silver bleeder valve on top of it to increase vacuum. Watch for foaming of both solutions at first and reduce vacuum (by turning the bleeder valve counterclockwise) to calm that as necessary. Standard degassing lasts ~8 minutes (flip vacuum switch to "Timer") but you can vary that by flipping the vacuum switch to "On" and timing the degassing yourself.

5. While things are degassing, open the Thermostat/Calibration window. Set temperature here at the actual run temperature you want. Adjust temperature with up and down arrows. Click Set Jacket Temp to set it. The instrument's temperature range is 2° to 80°.

6. Open ITC Controls window. Set Experimental Parameters as follows:

Total # injections	up to 500
Cell temperature	actual temp. you want

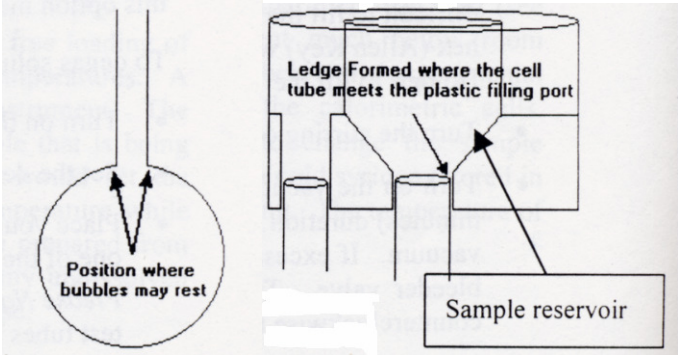
Reference Power	15 (middle of the range)
Initial delay	at least 60 sec
Syringe conc.	conc. of ligand (can add this later)
Cell conc.	conc. of protein (can add this later)
Stirring speed	310 is recommended (suspended particles will require higher speed)
Data file name	your choice.itc (unique characters in first ten)
Feedback Mode	high (for faster response times)
ITC Equil. Options	auto (machine will proceed on its own through equil. steps)

Set Injection Parameters as follows:

Volume	5 ul, maybe (see special injection notes at the bottom of this SOP)
Duration	10 sec (default is always 2X volume to allow time for return to baseline)
Spacing (between injections)	240 (typical range 180 - 300)
Filter period	2 (higher for slow thermal process)

Edit Mode
Can make different settings for first injection with Unique button

7. If it has been over a week since degassed water was put in the reference cell, load the reference cell with 1.8 ml of degassed water. Place the needle all the way at the bottom of the chamber then withdraw it back up ~1mm and leave it in place as you slowly add the water (i.e., don't withdraw the needle as you deliver water). Once the water comes up into the tube, stir the water in the chamber as much as you can with the needle to dislodge any air bubbles. Then remove the two air bubbles from the shoulders of the chamber using quick pressure to flush them out and once they are gone, remove excess volume from the cell by placing the delivery needle on the cell's inner ledge, drawing fluid out until you pull air out.



8. After rinsing the sample cell twice with ~2 ml of your buffer, load 1.8 ml of your protein sample into the sample cell as in Step 7.

9. To load your ligand into the ITC syringe, first put the tubing of the loading syringe onto the silver filling port. Click Open Fill Port on the lower right of the ITC Controls window. Place paddle in the tube of your degassed ligand with the tip almost at the bottom. Pull on the loading syringe to draw your ligand up into the ITC syringe. When the syringe is completely full and some ligand has come out into the loading tubing, click Close Fill Port. If there is a bubble at the very top of the ITC syringe, ignore it even if it seems large. MicroCal has shown that a bubble just below the plunger tip does not affect injection accuracy.

10. If you think there's a chance that bubbles may have been drawn into the ITC syringe along with your ligand, remove the loading syringe tubing and run the Purge-Refill function once or twice to remove them. If there were any, you'll see them appear in the ligand tube.

11. To remove air from the bottom of the paddle, set the Distance (in the pipette control window) to something tiny (like 0.01 in). Remove the ligand tube from the injector assembly then click Down to expel a tiny amount of liquid onto a Kimwipe. Do that twice.

12. Carefully insert the pipette into the sample cell access tube, the hole on the right. Near the bottom you have to push a little against a rubber O-ring to seat the assembly. Be sure the toothed wheel is right next to the stirrer platform. (You can wait until the machine reaches "ITC Pre-run" to insert the injector if you prefer, but that might cause the temp. to stay 1° higher than the set temp.)

13. Click on the Start flag to start the run. Check on the Display window to see that the Jacket and Sample temperatures have reached the desired levels. When they have, the red status letters change from "Heating" to "ITC Pre-run". The machine will then go into "Pre-stirring equilibration" mode. If it stays there for 20-30 minutes, hit Stop then Start again quickly and it will cycle through all its equilibrations but faster this time.

14. When it reaches "Final baseline equilibration" the stirrer will start. If at this point the lower right window (pipette control) goes gray, it means the syringe is not communicating so check the wire connection between the syringe and the box. You may need to wiggle that to make the connection and start again.

15. The next stage will be "ITC Pre-titration Delay". You can click on the Rescale to Show All button to see your whole trace. Finally, you will get to "ITC injection #1" and the experiment is off and running. You can calculate the approximate running time by multiplying your number of injections times the spacing time interval.

16. If you think you have enough injections, you can stop the ITC at any time by clicking on the STOP sign on the ITC Controls tab. You can also let it finish all

the injections you entered in the Method or let it keep injecting until it runs out of ligand.

17. To analyze your data, click on the MicroCal ITC Analysis icon. This opens a second version of Origin not interlaced with instrument control. Follow the instructions in the **Analyzing ITC Data** protocol on the website.

18. To clean up the machine for the next user, remove your sample and rinse the sample chamber with water. Then hook up the automatic washing system and put the injector in the loop so that detergent solution will pass through it as well as the sample chamber. Pull 200—250 ml of 5% Liqui-Nox through everything followed by an equal volume of water. Leave water in the sample chamber. Rinse out the injector with methanol (by hand) then pull air through it for 10-15 minutes using the vacuum set-up. Leave the paddle in air.

19. To close the VPViewer software, pull down from System and choose Quit Program. Closing the window from the X in the upper right-hand corner will result in your getting stuck in an infinite loop. Once the software is closed, you can turn off the ITC with the switch on the back. Leave the computer on.

Injection notes: Set your first injection to be 0.5 ul. This will expel the tiny bubble that forms at the tip of the syringe when you insert the syringe into the sample cell. This way your first full-size injection (the second one) will be more accurate. Delete the data from the first tiny injection before doing curve-fitting in Origin.

You should note how long it takes for the baseline to be reached again after the first tiny injection and program future runs to include that delay.

Updated: 5/21/07

Note: This protocol is a practical hands-on guide to using the instrument. Reading this is not a substitute for reading the manufacturer's manual, something we strongly recommend that you do.